PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12Q 1/68, 1/70, G01N 33/53

A1

(11) International Publication Number: WO 89/ 02934

(43) International Publication Date: 6 April 1989 (06.04.89)

(21) International Application Number: PCT/US88/03367

(22) International Filing Date: 30 September 1988 (30.09.88)

(31) Priority Application Number: 103,979

(32) Priority Date: 2 October 1987 (02.10.87)

(33) Priority Country: US

(71) Applicant: MICROPROBE CORPORATION [US/US]; 1725 - 220th Street S.E., Suite 104, Bothell, WA 98021 (US).

(72) Inventors: SCHWARTZ, Dennis, E. ; 20621 N.E. 37th Way, Redmond, WA 98053 (US). ADAMS, Trevor, H. ; 18440 N.E. 199th Street, Woodinville, WA 98072 (US).

(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN PAPILLOMAVIRUS TYPE DIAGNOSIS WITH NUCLEOTIDE PROBES

(57) Abstract

A rapid *in situ* hybridization assay for detecting the presence of Human Papillomavirus (HPV) in a non-frozen cellular smear fixed on a slide or other support in the absence of aldehyde-based cross-linking reagents. The assay can be conducted in less than four hours and utilizes readibly detectable nucleic acid probes, typically labelled by nick-translation with biotin, for binding to an avidin or streptavidin labelled enzyme, or with an isotope for visual detection.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BJ	Benin
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

HUMAN PAPILLOMAVIRUS TYPE DIAGNOSIS WITH NUCLEOTIDE PROBES

FIELD OF THE INVENTION

The present invention relates generally to the diagnosis of viral infections and, more specifically, to the detection of Human Papillomavirus using nucleic acid in situ hybridization assays.

BACKGROUND OF THE INVENTION

Prior to the 1940's, the leading cause of cancer death in women was cervical carcinoma. In 1943, Papanicolaou demonstrated that the cytology of stained exfoliated cells could be used for the detection of the early and late forms of cervical carcinoma. Since 1954, the "Pap smear" has been used as a cancer screening method and has been largely responsible for decreasing the mortality rate in women by cervical cancer in the Western World, such that it now ranks behind carcinoma of the breast, bowel, gastrointestinal tract, and ovary.

Cervical cancer is the result of a spectrum of morphological changes. The pre-invasive forms are mild dysplasia, severe dysplasia, and carcinoma in situ (these forms are also termed cervical intraepithelial neoplasia [CIN] and graded in their order of increasing severity as CIN I, CIN II and CIN III). The subsequent invasive forms are micro-invasive carcinoma and invasive carcinoma.

The Pap smear detects both pre-invasive and invasive forms of cervical cancer in a test which is inexpensive, easy to use, and for which cells are readily obtained. This test rarely gives false-positive results, but unfortunately gives high false-negative results; approximately 10-20% of abnormal Pap smears

are incorrectly reported as normal (Benedet, John L., and Murphy, Katherine J., "Cervical Cancer Screening," Postgraduate Medicine, 78:69-79 (1985)).

Since the late 1970's, evidence has been mounting that the primary etiological agent of cervical cancer is Human Papillomavirus (HPV) (Durst, M., Gissmann, L., Ikemberg, H., and zur Hausen, H.A., "Papillomavirus DNA from a Cervical Carcinoma and Its Prevalence in Cancer Biopsy Samples from Different Geographic Regions," Proc. Nat. Acad. Sci., 80:38812-38815 (1983), Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W., and zur Hausen, H.A., "New Type of Papillomavirus DNA: Its Presence in Genital Cancer Biopsies and in Cell Lines Derived from Cervical Cancer," EMBO J., 3:1151-1157 (1984), and Kreider, J.W., Howett, M.K., Wolfe, S.A., Bartlett, G.L., Zaino, R.J., Sedlacek, T.V., and Mortel, R., "Morphological Transformation In Vivo of Human Uterine Cervix with Papillomavirus from Condylomata Acuminata," Nature, 317:639-641 (1985)). More than 46 types of HPV have now been characterized, but at present only Types 6, 11, 16, 18, 31, 33, and 35 have been detected in the cervix. Of these, Types 6 and 11 are found in over 60% of cervical warts (Kreider, J.W., et al., supra, and Gissmann, L., Wolnik, L., Ikenbert, H. Koldovsky, U., Schnurch, H.G., and zur Hausen, H., "Human Papillomavirus Types 6 and 11 DNA Sequences in Genital and Laryngeal Papillomas and in Some Cervical Cancers," Proc. Natl. Acad. Sci., 80:560-563 (1983)), whereas Types 16 and 18 are found in over 90% of tissues characterized as being pre-invasive or invasive forms of cervical cancer (Durst, M., et al., supra, Boshart, M., et al., supra, McCance, D.J., and Clarkson, P.K., "Prevalence of Human Papillomavirus Type 16 DNA Sequences in Cervical Intraepithelial Neoplasia and Invasive Carcinoma of the Cervix," British Journal of Obstetrics and Gynecology, 92:1101-1105 (1985), and

Pater, M.M., Dunne, J., Hogan, G., Ghatage, Prafull, and Pater, A., "Human Papillomavirus Types 16 and 18 Sequences in Early Cervical Neoplasia," Virology, 155:13-18 (1986)). HPV Types 31, 33, and 35 (and other related/unclassified HPV types) are found in the remaining 10% of cervical cancers (Lorincz, A.T., Lancaster, W.D., Kurman, R.J., Jenson, A.B., and Temple, G.F., "Characterization of Human Papillomaviruses in Cervical Neoplasias and Their Detection in Routine Clinical Screening," "Viral Etiology of Cervical Cancer," Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, Branbury Report, 21:225-237 (1986) and Cole, S.T., and Streeck, R.E., "Genome Organization and Nucleotide Sequence of Human Papillomavirus Type 33, Which is Associated with Cervical Cancer," J. Virology, 58.3:991-995 (1986)). One current hypothesis is that HPV-6 and HPV-11 are associated with benign growths (wart lesions) within the cervix (or vulva or vagina), whereas HPV Types 16, 18, 31, 33, and 35, and the related or as yet unclassified HPV types are implicated as etiological agents of cervical cancer.

Cervical cancer may be a sexually transmitted disease. The disease is absent in sexually inactive women, but does occur with high frequency among sexually active women, particularly those who are sexually active at an early age and who have or have had multiple sex partners. Consistent with these observations, HPV-induced warts are observed, although rarely, on the penile shaft or urethra of males, and HPV is found in sperm (Ostrow, R.S., Zachow, K.R., Niimura, M., Okagaki, T., Muller, S., Bender, M., and Faras, A.J., "Detection of Papillomavirus DNA in Human Semen," Science, 231:731-733 (1986)).

Techniques for the detection of HPV in human cells use either antibodies or DNA probes. Antibodies have been difficult to develop since there is no culture system for propagating HPV, and thus no readily

available method for preparing the viral proteins. HPV proteins recently have been prepared in E. coli expression systems, thus allowing the generation of HPV-specific polyclonal or monoclonal antibodies (Matlashewski, G., Banks, L., Wu-Liao, J., Spence, P., Pim, D., and Crawford, L., "The Expression of Human Papillomavirus Type 18 E6 Protein in Bacteria and the Production of Anti-E6 Antibodies," J. Gen. Virol., 67:1909-1916 (1986)). However, it is recognized that antibodies may never be adequate for the diagnosis of HPV infections since detectable HPV antigens are typically not found in the later stages of HPV infection (Nakajima, T., et al., "The Frequency of Papillomavirus Infection in Cervical Precancerous Lesions in Japan: An Immunoperoxidase Study," Jpn. J. Cancer Res., 77:891-895 (1986)). In contrast, it has been alleged that HPV can be detected by DNA probes at all stages of infection; thus, this is becoming a preferred diagnostic method (Schneider, A., Kraus, H., Schumann, R., and Gissmann, L., "Papillomavirus Infection of the Lower Genital Tract: Detection of Viral DNA in Gynecological Swabs," Int. J. Cancer, 35:443-448 (1985) and Burk, R.D., Kadish, A.S., Calderin, S., and Romney, S.L., "Human Papillomavirus Infection of the Cervix Detected by Cervicovaginal Lavage and Molecular Hybridization: Correlation with Biopsy Results and Papanicolaou Smear," Am. J. Obstet. Gynecol., 154:982-989 (1986)).

Recent DNA probe-based studies have indicated a wide and disturbing variance in HPV infection within the U.S. population. An investigation conducted in the Seattle, Washington, U.S.A., area indicates an HPV incidence of 6% and 13%, within suburban upper class women and urban lower class women, respectively. At present, no such studies have been reported on the prevalence of HPV infections within male populations.

The strong association of cervical cancer with specific HPV viral types indicates a need for a

nucleic acid probe-based diagnostic test that distinguishes between the benign and oncogenic forms of this virus. First, a DNA probe test could be used as a secondary test, after a positive Pap smear, to identify the type of HPV within a clinical sample. Second, such a test could be used in conjunction with the Pap smear as a screening tool for the routine concurrent detection of abnormal and HPV infected cervical cells. Finally, the DNA probe test could ultimately displace the Pap smear test, if HPV is proven unequivocally to be the etiological agent of cervical cancer and a rapid, reliable and economical HPV assay capable of utilizing a cervical smear format were commercially available.

Some proponents in the medical community contend that a test for the presence and type of HPV within cervical cells could have a dramatic impact on the early detection of cervical cancer and on the choice of therapeutic treatments for these cancers. In addition, a rapid diagnostic HPV test would allow the detection and typing of HPV infections within the male population. For both men and women, a specific and sensitive test for HPV could significantly curb the spread of this viral infection through appropriate counseling and/or through the use of emerging therapeutic agents.

SUMMARY OF THE INVENTION

The present invention provides a rapid in situ hybridization assay for detecting the presence of or typing of Human Papillomavirus (HPV) in a biological sample, such as a non-frozen cellular smear that has been fixed on a support in the absence of aldehyde-based cross-linking reagents, the assay including the steps of:

combining the nucleic acids from the fixed biological sample with at least one detectable probe, preferably nick-translated with a detectable nucleotide of about 50 or more nucleotides, or analogs thereof,

wherein the probe is capable of specifically hybridizing with a substantially complementary region from one or more HPV types; and

detecting the presence or absence of probe hybridization complexes.

Importantly, the entire assay can be completed in less than about 4 hours, preferably about 2 hours, and most preferably less than about 2 hours. The assay is particularly useful for the detection of one, two or more different HPV types, including 6, 11, 16, 18, 31, 33, and 35.

The biological sample, for example a cervical smear, is typically first fixed, such as by treatment in an alcohol bath, onto a support, such as a glass slide. Preferably, the assay utilizing a cervical smear sample fixed on a glass slide will comprise the steps of: (i) inactivating competing endogenous enzyme activity; (ii) denaturing nucleic acids in the sample; (iii) hybridizing a detectable probe to the target nucleic acids, wherein the probe comprises a sequence of 200 to 600 or more nucleotides complementary to one HPV type DNA or mRNA; (iv) washing the sample to remove unbound probes; (v) incubating the sample with the detection agents; and (vi) visually inspecting the sample, such as with the aid of a microscope.

The assay of the present invention may be provided in kit form. For example, a typical kit will include a first probe reagent component comprising a biotin-labelled probe of at least 50 nucleotides complementary to a nucleic acid sequence of HPV types 6 or 11 and/or a second probe of at least about 50 nucleotides complementary to nucleic acids sequence of HPV types 16, 18, 31, 33, or 35; a denaturation reagent for converting double stranded DNA to single stranded DNA; and a hybridization reaction mixture. The kit can also include an avidin-labelled or streptavidin-labelled enzyme and a substrate for the enzyme.

Other features and advantages of the invention will become apparent from the following detailed description, which describes the present invention by way of example.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 shows partial nucleotide sequence comparisons between a Human Papillomavirus (HPV) Type associated with benign cervical warts (HPV-6) and two HPV Types believed to be the etiologic agents of cervical carcinoma (HPV-16 and HPV-33). About 700 nucleotides of sequence at the 5' end of each HPV genome are shown as follows: Figure 1a shows HPV-6 compared with HPV-11; Figure 1b shows HPV-6 compared with HPV-16; Figure 1c shows HPV-6 compared with HPV-33; and Figure 1d shows HPV-16 compared with HPV-33.

DETAILED DESCRIPTION

In accordance with the present invention, nucleic acid probes of at least about 50 nucleotides are used in rapid, reliable and economical in situ hybridization assays for detecting the existence and type of HPV present in biological samples, such as cervical or other cellular smears. The assays can be streamlined such that the entire test can be conducted under preselected hybridization conditions with a non-isotopic format in less than about four hours, and as little as about two hours or less, with few steps. In addition, all steps may be performed at room temperature, thus alleviating the need for temperature-controlled incubators.

The in situ hybridization tests of the present invention, when using, e.g., unstained smears of cervical cells, are often more sensitive than the Southern blot "gold standard," particularly when conducted on slides, which allows for the detection of a few positive cells in a milieu of negative cells.

Furthermore, these assays can be conducted in large batches in short time periods, rather than the several days typically required in hybridization formats using the Southern blot or dot blot methods.

In a preferred embodiment, the biological sample to be tested includes cell smears obtained by standard techniques, such as scraping (e.g., a cervical smear) or biopsy samples converted to smears (such as with the Cyto-Trac system, Medical Packaging Corporation, Panorama City, CA). Sources of cells include cervical, vaginal, vulval, oral, prostate, lung, rectal or any body tissue suspected of containing HPV.

Typically, cellular smear samples are collected and fixed to a support, such as a glass surface (e.g., a glass slide), plastics (e.g., polycarbonate), or other transparent inert substrates. Fixing agents may be precipitants, such as picric and mecuric acid, ethanol, ethanol/acetic acid, methanol and methanol-acetone mixtures. Most preferred precipitating fixation solutions include ethanol and Carnoy's B solution. Standard aldehyde-based fixation is generally unnecessary.

Pretreatment of the cells to increase probe diffusion may be helpful, and can include acid treatment or protease treatment. When utilizing an enzyme as the detecting agent, this pretreatment may also serve to inactivate endogenous enzyme activity.

Various in situ hybridization protocols for detecting viral infections are known in the art, and can be utilized in accordance with the teachings of the present invention to assay for HPV in the cellular smears. The following two review articles provide an overview of in situ hybridization technology: Singer, R.H., et al., Biotechniques, 4(3):230-250 (1986), and Haase, A., et al., Methods in Virology, Vol. VII, pp. 189-226 (1984), and are both incorporated by reference herein.

Target polynucleotides can be obtained from a wide variety of sources, depending upon the particular HPV type to be detected. By way of example, such probes can be any HPV specific nucleic acid sequences integrated or otherwise present in a biological sample, including mutations of the wild-type virus populations, regardless of whether they are phenotypically expressed.

Probes are DNA or RNA polynucleotides or oligonucleotides, or their analogs, which have sufficient complementarity with the target polynucleotides so that stable binding occurs between target and probe. Homoduplexing is preferred, i.e., a perfect base match, but such is often not achieved when using cocktails of long probes and/or when detecting multiple HPO types. The degree of homology required for detectable binding varies with the stringency of the hybridization medium and/or wash medium.

The lengths of the probes which are useful for the given invention are at least 15 bases, but may be 50 to 100 bases long, preferably 200 to 600 bases or more. Essentially whole HPV virus genomes of about 8,000 nucleotides, with or without plasmid vector sequences, are commonly utilized for nick-translation in the presence of labelled nucleotides to yield an average probe length of about 400 bases.

DNA probes may be cloned in bacterial host cells following insertion into appropriate replication vectors, such as pBr322 or M13, or vectors containing RNA polymerase specific promoters, such as the SP6 promoter, and purified from the host cell by cell lysis, DNA extraction. Further purification, if desired, may be achieved by digestion with selected restriction enzymes, and further separation by gel or column fractionation techniques.

The probes used in the present invention may also be synthesized, chemically or enzymatically, using

commercially available methods and equipment. For example, the solid phase phosphoramidite methods are particularly useful for producing smaller probes. (See, Caruthers, et al., Cold Spring Harbour Symp. Quant. Biol., 47:411-418, 1982, and Adams, et al., J. Am. Chem. Soc., 105:661, 1983, both of which are incorporated herein by reference). DNA probes can also be synthesized, for example, by reverse transcription of mRNA or produced by nick-translation of cloned HPV genes.

Nucleotide analogs within HPV specific sequences that can be inserted during chemical or enzymatic synthesis include: 1-(2-deoxy-a-D-ribofuranosyl)-2-pyrimidinone, 2'-deoxyinosine, 2'-deoxy-7-deazaguanosine, 2'-deoxy-5-substituted uridine, or the appropriately blocked phosphoramidites thereof. These can be substituted for naturally occurring nucleotides, e.g., during labelling or synthesis of the probe, yet maintain acceptable hybridization specificity of the probe. Other analogs having similar functionality can, of course, be produced in accordance with well known teachings in the art.

When synthesizing a probe for a specific target, the choice of sequence will determine the specificity of the test. For example, by comparing DNA sequences from several virus isolates, one can select a sequence for virus detection that is either type specific or genus specific. Comparisons of DNA regions and sequences can be achieved using commercially available computer programs. Generally, the more unique the sequence selected to be probed, the less background noise will be generated.

Probes may be labeled by any one of several methods typically used to detect the presence of hybrid polynucleotides. A common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{34}S, ^{14}C, or ^{32}P labeled probes or the like. Other labels include

directly conjugated fluorophores, chemiluminescent agents, enzymes, and enzyme substrates. Alternatively, the same components may be indirectly bonded through a ligand-antiligand complex, such as antibodies reactive with a liquid conjugated with label. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The choice of label dictates the manner in which the label is incorporated into the probe. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes, for example, by using DNA synthesizers, by nick-translation, by tailing of radioactive bases to the 3' end of probes with terminal transferase, by copying M13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive dNTP's, or by transcribing RNA from templates using RNA polymerase in the presence of radioactive rNTP's.

Non-radioactive probes can be labeled directly with a signal (e.g., fluorophore, chemiluminescent agents or enzyme) or labelled indirectly by conjugation with a ligand. This ligand then binds to a receptor molecule which is either inherently detectable or covalently bound to a detectable signal, such as an enzyme or photoreactive compound. Ligands and antiligands may be varied widely. Where a ligand has a natural "antiligand", namely ligands such as biotin (which is recognized by avidin or streptavidin), thyroxine, and cortisol, it can be used in conjunction with labeled, naturally occurring receptors. Alternatively, any haptenic or antigenic compound can be used in combination with a suitably labelled antibody. A preferred labelling method utilizes biotin labelled analogs of polynucleotides, as disclosed in

Langer, P. and Waldrop, A. (Proc. Nat. Acad. Sci. U.S.A. 78: 6633-6637 1981), which is incorporated herein by reference. Another preferred labelling method utilizes a direct conjugation of enzyme (e.g., horseradish peroxidase or alkaline phosphatase) to the DNA in DNA probe (either double-stranded or single-stranded). See, Reng, M. and Kurz, C. "A Colorimetric Method for DNA Hybrization," Nucl. Acids Res., 12:3435-3444 (1984), which is incorporated herein by reference.

Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases, ureases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescers include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol.

The amount of labeled probe present in the hybridization solution may vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the cellular target nucleic acid, and the precise stringency of the hybridization medium and/or wash medium. Generally, substantial probe excesses over the stoichiometric amount of the target will be employed to enhance the rate of binding of the probe to the target nucleic acids.

Prior to adding the hybridization solution and the labelled probe to the cells, potentially competing endogenous enzyme activity (i.e., those with overlapping activity to the signal detection system of the probe) may be inactivated. Typically, this is accomplished by mixtures of ethanol and acetic acid, but any of a variety of well known inhibiting agents may be utilized.

Hybridization solutions and procedures are generally described by Gall and Pardue (1969), Proc.

Natl. Acad. Sci., U.S.A., 63:378-383; John, Burnsteil and Jones (1969) Nature, 223:582-587; and "Nucleic Acid Hybridization: A Practical Approach", Eds. Hames, B. and Higgins, S. IRL Press (1985) Washington, D.C., all of which are incorporated herein by reference. As improvements are made in hybridization techniques, they can readily be applied.

Various hybridization solutions may be employed provided they provide rapid hybridization in accordance with the present invention, i.e., typically less than two to three hours, preferably less than one hour, and most preferably, 20 to 30 minutes. These solutions can comprise from about 20 to 60% volume, preferably about 30%, of a polar organic solvent. A common stringent hybridization solution employs about 50% formamide, about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris, HEPES or PIPES, about 0.05 to 0.2% nonionic or ionic detergent, such as Tween 20 or sodium dodecylsulfate, or minor amounts of EDTA, Ficoll (about 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabelled carrier nucleic acids from about 0.1 to 5 mg/ml, fragmented DNA, e.g., calf thymus or salmon sperm DNA, and/or yeast tRNA or partially fragmented rRNA, and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents, which include a variety of water-soluble or swellable agents, such as anionic polymers of polyacrylate or polymethacrylate, charged saccharide polymers, such as dextran sulfate (preferably, about 10%), and tetra-alkylammonium salts or triethlamine salts. (See Melchor, W. B. and Von Hippel, P. H., "Alteration of the Relative Stability of dA.dT and dG.dC Base Pairs in DNA," Proc. Nat. Acad. Sci. USA, 70: 298-302 (1973) and

Orosz and Wetmar, Biopolymers, 16:1183-1199 (1977), which are both incorporated herein by reference.)

Stringent hybridization conditions are preferred either during the hybridization or wash step. The precise degree of stringency typically is controlled by ionic strength, partially denaturizing solvents and temperature. The stringency of hybridization or washing is conveniently varied by changing the polarity of the reactant solution through manipulation of the concentration of a partially denaturizing solvent, such as formamide, within the range of about 20% to 50%, typically the higher percentages. Stringency can also be conveniently varied by changing salt concentration, up to 0.5 M or higher, preferably 10 mM to 150 mM. Strigency can also be varied by changing temperatures, which will normally be in the range of about 20° to 75°C, but the present invention preferably utilizes temperatures from about 20° to 37°C. By experimentation, following the teachings of the present invention, one can define conditions which permit hybridization at room temperature within a particular assay format.

After the cells fixed to the support have been contacted with a hybridization solution, the cello are then typically introduced into a wash solution having predetermined concentrations of salts, buffers, and detergents. The time period for the wash may vary from five minutes to an hour or more. Typically, it is the wash solution that most often determines the stringency and facilitates dissociation of mismatched duplexes. After washing the hybridization complex at room temperature with dilute buffered sodium chloride solution, the complex may be assayed for the presence of duplexes (e.g., bound probe) in accordance with the nature of the label. This detecting step will typically be completed in from one to three hours, but

may be completed in 15 to 30 minutes depending on the detection system.

Where the label is radioactive, the support is dried and exposed to X-ray film. In the alternative, a nuclear track emulsion is coated upon the support in the dark, allowed to develop, washed, stained, and viewed under a microscope (Haase, et al., Methods of Virology, Vol. VII, pp. 209-210).

Enzymatic detection is typically performed in conjunction with biotin, such as biotinylated peroxidase or alkaline phosphotase. Enzyme-conjugated avidin or streptavidin are then used to bind the enzyme to the probe. After the appropriate enzyme substrate has been added, the cells may be observed visually for the presence of HPV.

Another embodiment of the present invention comprises kits comprising compartments (e.g., vials) containing the means components for performing the assays of the present invention. In a preferred embodiment, the kit includes a first probe reagent comprising a probe of at least about 200 to 600 nucleotides complementary to a nucleic acid sequence of at least one HPV type (e.g., 6 or 11) and/or a second probe of at least about 200 to 600 nucleotides complementary to a nucleic acid sequence of one or more different HPV types (e.g., 16, 18, 31, 33 an 35); and a denaturation step (preferably heat or a chemical denaturation reagent) for converting double stranded DNA in cells to single stranded DNA. The kit can also include an avidin or streptavidin labelled enzyme and a substrate for the enzyme, as well known in the art. All of the kit compositions are commonly provided in liquid form or may be lyophilized for subsequent reconstitution with water at the assay site.

The following examples are offered by way of illustration and not by limitation.

EXPERIMENTAL

Reagents

DNA probes were synthesized on an Applied Biosystems DNA Synthesizer (Model 380B, Foster City, CA) with reagents from the same source. DNA probe purification was accomplished by: 1) fractionation of the tritylated product with a high pressure liquid chromatography system using a reverse phase column, or 2) fractionation by gel electrophoresis on a 20% polyacrylamide gel containing 7M urea. Computer analysis of HPV nucleotide sequences was done with the Micro-Genie Program (Beckman Instruments, Palo Alto, CA).

Molecular clones containing HPV Types 3a, 6b, 6vc, 8, 8c, 10, 11a, 12, 13, 14a, 15, 16, 17a, 18, 19, 20, 21, 22, 23, 24, and 25, were obtained from laboratories in Japan, Europe and the U.S. The CaSki and A-549 cell lines were obtained from the American Type Culture Collection.

SSC buffer is 0.015M sodium citrate (pH 7.0), 0.15M NaCl; PBS is 0.01M sodium phosphate (pH 7.4), 0.13M NaCl; and Denhardt's solution is 0.02% ficoll 400, 0.02% polyvinylpyrolidone (MW 360,000), 0.02% BSA. Carnoy's B solution is 10% HOAc, 30% chloroform, 60% EtOH. HRP substrate solution is 0.4 mg/ml aminoethyl carbazole, 0.025% hydrogen peroxide, 0.1M NaOAc (pH 4.5).

I. CLONING OF HPV GENOMES

a. Source of DNA.

Cells from patients were collected by cervical lavage, washed, lysed, and the nucleic acid isolated (Burk, R.D., Kadish, A.S., Calderin, S. and Romney, S.L., Am. J. Obstet. Gynecol. 154:982-989. 1986). HPV genomes were detected and typed by Southern blot by digesting aliquots of the DNA preparations with PstI, a restriction enzyme which cleaves the genomes of the different virus types into characteristic fragment

sizes. The digested DNA's were electrophoresed through agarose gels and then transferred onto nitrocellulose filters (Southern, E., J. Mol. Biol. 98:503 (1973). Filters were then probed with a mix of nick-translated HPV genome Types 6, 11, 16, and 18, washed at low stringency (Lorincz, A.T., Lancaster, W.D., and Temple, G.F., J. Virol. 58:225-229) and developed by autoradiography. DNA was selected for cloning based on HPV type, copy number, and episomal state.

b. HPV sequences.

The following references describe sequences of HPV Types 6, 11, 16, 18, 33: Schwarz, E., et al., "DNA sequence and genome organization of genital human papillomarvirus Type 6b", EMBO J. 2:2361-2368 (1983). Seedorf, K., et al., "Human papillomavirus type 16 DNA sequence", Virology 145:181-185, (1985); Dartmann, K., et al., "The nucleotide sequence and genome organization of human papillomavirus Type 11," Virology 151:124-130, (1986); Cole, S.T., and R.E. Streek, "Genome organization and nucleotide sequence of human papillomavirus Type 33, which is associated with cervical cancer," J. Virol. 58:991-995, (1986); and Cole, S.T. and O. Danos, "Nucleotide sequence and comparative analysis of the human papillomavirus Type 18 genome," J. Mol. Biol. 1983:599-608 (1987); all of which are incorporated herein by reference.

c. Cloning of HPV genome Types 6, 11, 16 and 18.

Approximately 10 μg of DNA containing Types 6, 11, or 16 were cut with BamH1; DNA containing Type 18 was cut with EcoR1. DNA's were then extracted with phenol:chloroform (1:1), chloroform, and then ethanol precipitated. Approximately 2-5 μg of each DNA were analyzed by Southern blot for hybridization to the appropriate gamma-32P-ATP labelled 24-mer oligonucleotides (see Table I below). Oligonucleotides contained approximately 50% G and C residues. Hybridizations were carried out at 43°C, 15 h, in oligo hybridization

solution (0.5 to 1.0 ng/ml oligo, 0.6M NaCl, 90 mM Tris HCl, pH8, 10 mM EDTA, 0.5% 5 x Denhardts, 30% formamide, 0.1 mg/ml hydrolyzed yeast RNA). Filters were washed 15 min, room temperature, in 1x oligo wash solution (0.09M NaCl. 0.009 M Tris, pH8, 0.6 mM EDTA, 0.1% SDS), 15 min at 50°C in 1x wash solution, and then 2 min, room temperature, in 1x wash solution. Probe hybridization was detected by autoradiography. In almost all cases, the HPV genomes detected were full length and complementary to the appropriate oligonucletides.

The remaining half of each sample, approximately 2-5 μg, was ligated to 1 μg, lambda DASH/R1 for Type 18 or DASH/BAM for Types 6, 11, and 16 (Stratagene, San Diego, CA). The reactions were packaged (Stratagene's Gigapack-Plus) and titered on E. coli P2392, a host which selects for recombinant phage. Approximately 3 x 10^5 plaque forming units were screened by filter hybridization (Maniatis, T., Fritsch, E.F., and Sambrook, J., Molecular cloning: a laboratory manual. 1982 by Cold Spring Harbor Laboratory). Hybridization and washing conditions were as established above for Southern blots. Positive clones were detected at a frequency of about one in 10^3.

TABLE 1

	Probe Sequence	HPV Type
1.	5'GGTTGAACCGTTTTCGGTCCCTCC 3'	6/11
2.	5-GAGTTACAGGACTAAAGGGTGTTC 3'	6/11
3.	5'CTGTCACATCCACAGGAACAGGTC 3'	6/11
4.	5'CAGAATAGCCATATCCACTGTCCG 3'	6/11
5.	5'GTGGTATCTACCACAGTAACAAAC 3'	6/11
6.	5'CTTCAGGACACAGTGGCTTTTGAC 3'	16
7.	5'GAAGCGTAGAGTCACACTTGCAAC 3'	16
8.	5'CAACGCATGTGCTGTCTCTGTTTC 3'	16
9.	5'CACTTCCACTACTGTACTGACTGC 3'	16
10.	5'GTCTCCATCAAACTGCACTTCCAC 3'	16
11.	5'CTGTGCAACAACTTAGTGGTGTGG 3'	16
12.	5'CAGACACACAAAAGCACACAAAGC 3'	16
13.	5'CAGTACGCCTAGAGGTTAATGCTG 3'	16
14.	5'CTAGAATTAGAGAATTAAGAGATT 3'	18
15.	5'GCGGTGCCAGAAACCGTTGAATCC 3'	18
16.	5'TCGTCGGGCTGGTAAATGTTGATG 3'	18
17.	5'GAATGCTCGAAGTCGTCTGCTGAG 3'	18
18.	5'AATGTCTTAATTCTCTAATTCTAG 3'	18
19.	5'GGATTCAACGGTTTCTGGCACCGC 3'	18
20.	5'CCTGTCGTGCTCGGTTGCAGGACG 3'	18
21.	5'ATTTTGGGGCTCTAAATGCAATAC 3'	18

d. Cloning of Types 31, 33, and 35.

Approximately 10 μg of DNA containing Type 31 or 35 were cut with EcoR1: DNA containing Type 33 was cut with BglII. DNAs were extracted with phenol:chloroform (1:1), chloroform, and then ethanol precipitated. Approximately half of each sample was analyzed by Southern blot for hybridization to nick-translated HPV16 DNA. Hybridizations were carried out at low stringency (30% formamide, 0.6M NaCl, 90 mM Tris, pH8, 10 mM EDTA, 0.5% SDS, 5 x Denhardts, 0.1 mg/ml yeast RNA, at 43°C). Filters were then washed 15 min, room temperature, 1 x wash solution (0.2 x SSC, 0.1% SDS where 1 x SSC is 0.15M NaCl, 0.015M sodium citrate), then 15 min, 50°C, 1 x wash solution, followed by 10 min. 65°C, 1 x NT wash solution. Probe hybridization was detected by autoradiography. Filters were then rewashed at higher stringency (0.03 x SSC, 0.1% SDS, 65°C, 15 min) and analyzed by autoradiography. In all cases, HPV genomes were full length and related but not identical to Type 16.

The remaining half of each sample was then ligated to the appropriate lambda DASH vector, and was packaged and titered as described above. Approximately 3×10^5 plaque forming units were screened by filter hybridization. Hybridization and washing conditions were as established above for the Southern blots.

e. Analysis and subcloning of HPV clones.

Bacteriophage DNA was isolated from positive clones and analyzed by Southern blot following restriction with PstI, BamH1, or EcoR1. In almost all cases, the clones also contained additional inserts which consisted of human genomic DNA. The DNA's were then cut with restriction enzymes which excised the HPV genomes away from the lambda and human DNA. EcoR1 was used for Types 18, 31, and 35; BamH1 was used for Types 6, 11, and 16; BglII was used for Type 33. The restricted DNA's were electrophoresed on agarose gels and the HPV

DNA isolated by eluting onto NA-45 paper (Schleicher and Schuell, Keene, NH), according to standard protocol.

The genomes were ligated to Bluescript M13 DNA (Stratagene) which had been EcoR1 or BamH1 digested and treated with calf intestinal phosphatase (Boeringer Mannheim, Indianapolis, IN). Bluescript vectors have large polylinkers with 26 unique restriction sites. The polylinkers are flanked by T7 and T3 polymerase promoters present at the NH_2-terminal portion of a LacZ gene fragment. The vectors also contain a 454 nucleotide intergenic region (M13 related). The ligation reactions were transformed into E. coli BB-4 (Stratagene) and recombinant colonies identified by restriction analysis of their DNA (Maniatis et al., above). With the exception of HPV6, all clones matched published restriction maps. The HPV6 clone did not have the expected 5.3 kb PstI fragment, but instead had 3.6 and 1.7 kb fragments. Recombinant plasmids contained full length HPV genomes and were free of human chromosomal DNA.

II. IN SITU HYBRIDIZATION WITH LONG DNA PROBES ON CULTURED CELLS

a. Preparation and Fixing of Cells on Glass Slides.

Mammalian cells (about 10,000) were spotted on glass slides in 100 ul of the appropriate culture media and grown at 37°C for 18-24 hrs. This method allowed the generation of a large number of slides, each containing about 50,000 cells that had tenaciously adhered to the glass surface.

Prior to in situ hybridization, the slides were dipped twice in PBS solution and then fixed in Carnoy's B (10% HOAc, 30% chloroform, 60% EtOH) solution for 3 min. The slides were blotted lightly to remove excess solution, air dried for 10 min., and

stored at -20°C until use. This treatment produced fixed cells with good morphology, high stability and with high accessibility of short and long probes to cellular DNAs during in situ hybridization.

b. In Situ Hybridization with Long Cloned DNA Probes.

Labeling of long probes (i.e., greater than 50 bases) with 32p was accomplished by nick-translation (Rigby, P., Rhodes, D., Dieckmann, M., and Berg, P., "Labeling Deoxyribonucleic Acid to High Specific Activity In Vitro by Nick Translation with DNA Polymerase I," J. Mol. Biol., 113:237-251 (1977)), and labeling with biotin was accomplished by nick-translation using biotinylated dUTP (Langer, P.R., Waldrop, A.A., and Ward, D.C., "Enzymatic Synthesis of Biotin-labeled Polynucleotides: Novel Nucleic Acid Affinity Probes," Proc. Nat. Acad. Sci., 78:6633-6637 (1981)). Thirty microliters of long probe hybridization solution (20mM PIPES at pH 7.4, 500mM NaCl, 0.05% NaPPi, 50% formamide, 1 x Denhardt's solution, 10% dextran sulfate, 200 mg/ml calf thymus DNA, 200 mg/nl yeast RNA and 10 ng of 32P- or biotin-labeled long probe) was layered over the cells fixed on glass slides. A siliconized coverslip was placed over the solution, and the samples were incubated in a humidified chamber for 1 hr. at 37°C. Following removal of the coverslip by soaking in 2 x SSC, the sample was stringently washed twice (5 min./wash) in 2 x SSC at room temperature.

Detection of 32P-labeled probe was accomplished with autoradiography and of biotin-labeled probe by either of the two following methods:

Method #1: A conjugate of horse radish peroxidase (HRP) with streptavidin (75 ul) was added to the sample and incubated for 30 min. at room temperature. The sample was washed in 2 x SSC for 5 min., then in 2 x SSC, 0.1% triton X-100 for 5 min. The sample was very lightly blotted, and 75 ul HRP substrate

solution was added and incubated for 30 min. at room temperature. The sample then was washed with PBS for one minute and mounted for viewing under a microscope.

Method #2: (About twofold more sensitive than Method #1, but requires more steps). The sample is treated with 75 ul of blocking solution (10% normal rabbit serum, 4% BSA, 2 x SSC) for 10 min. at 37°C and washed twice in 2 x SSC at room temperature. Biotinylated goat anti-avidin IgG (75 ul at mg/ml in 0.1% BSA) is added, incubated for 30 min. at 37°C, and removed by three washes (3 min./wash) in 2 x SSC at room temperature. Finally, 75 ul of an avidin-biotinylated HRP complex (ABC complex, Vector labs) was added and incubated for 15 min. at 37°C. Following three washes (3 min./wash) in 2 x SSC at room temperature, 75 ul of substrate solution was added, incubated for 30 min. in the dark at room temperature, and removed by rinsing in PBS for 1 min. The sample was lightly blotted and mounted for viewing.

III. RESULTS

a. Computer Analysis of Known HPV Nucleotide Sequences

The known genomic nucleotide sequences of HPV Types 1a (Danos, O., Katinka, M., and Yaniv, M., "Human Papillomavirus 1a Complete DNA Sequence: A Novel Type of Genome Organization Among Papovaviridae," EMBO Journal, 1.2:231-236 (1982)), 6b (Schwarz, E. et al., "DNA Sequence and Genome Organization of Genital Human Papillomavirus Type 6b," EMBO Journal, 2.12:2341-2348 (1983)), 8 (Fuchs, P.G., et al., "Epidermodysplasia Verruciformis - Associated Human Papillomavirus 8: Genomic Sequence and Comparative Analysis," J. Virology, 58.2:626-634 (1986)), 11 (Dartmann, K., et al., "The Nucleotide Sequence and Genome Organization of Human Papillomavirus Type 11," Virology, 151:124-130 (1986)), 16 (Seedorf, K., et al., "Human Papillomavirus

Type 16 DNA Sequence," Virology, 145:181-185 (1985)), 33 (Cole, S.T., and Streeck, R.E., supra), and the known regions of Type 18 (Matlashewski, G., et al., supra, and Schneider-Gadicke, A.S., and Schwarz, E., "Different Human Cervical Carcinoma Cell Lines Show Similar Transcription Patterns of Human Papillomavirus Type 18 Early Genes," EMBO Journal, 5.9:2285-2292 (1986) and Cole, S. T. and Denos, O., "Nucleotide Sequence and Comparative Analysis of the HPV Type 18 Genome," J. Mol. Biol., 1 93:599-608 (1987)) have been cross-compared using the MicroGenie Program (Beckman Instruments). In part, this was to determine the extent of nucleotide sequence divergence among the various HPV types and to determine how this divergence would affect the strategy of preparing highly specific synthetic DNA probes. It was found that each type of HPV had undergone surprising divergence in nucleotide sequence as shown in Figure 1, a-d, where HPV Types 6 and 11, 6 and 16, 6 and 33, and 16 and 33 are compared. (Only about 700 nucleotides from the 5' end of each virus is shown.) It is clear that there is considerable sequence conservation between Types 6 and 11. In contrast, there is much less sequence conservation between HPV Types 6 and 16, 6 and 33, and 16 and 33. Overall throughout their entire genomes, Types 6 and 11, 6 and 16, 6 and 33, and 16 and 33 are 82%, 59%, 58%, and 66% homologous, respectively.

b. Demonstration of In Situ Hybridization

A model system for demonstration and optimization of in situ hybridization is the CaSki cell line which is derived from a cervical tumor and contains multiple copies of HPV-16 (Yee, et al., "Presence and Expression of Human Papillomavirus Sequences in Human Cervical Carcinoma Cell Lines," Am. J. Pathology, 119:361-365 (1985) and Pater, M.M., and Pater, A., "Human Papillomavirus Types 16 and 18 Sequences in Carcinoma Cell Lines of the Cervix," Virology,

145:313-318 (1985)). CaSki cells are easily cultured and therefore ideal for the production of a large number of "standardized" glass slides containing immobilized cells. Such standardized samples are extremely useful for optimization of in situ hybridizations. It was found that a large number of standardized glass slides could be prepared conveniently by growing cells directly on glass slides overnight. During this growth, the cells adhered tightly to the slides and subsequently could be processed using a variety of procedures without cell loss.

After overnight growth, slides with immobilized CaSki cells were fixed in Carnoy's B solution for 5 min., rinsed in EtOH, air dried and stored at -20°C until use. A variety of procedures were tested, including fixing with paraformaldehyde or glutaraldehyde. Carnoy's B fixative was found to be superior for 1) maintaining morphology of the cell, and 2) giving a more clearly defined colored precipitate when detecting HPV DNA using a horseradish peroxidase (HRP) detection system.

Effective in situ hybridization assays were demonstrated using radioactively labeled DNA probes. Non-isotopic hybridizations were also conducted with biotinylated long probes using modifications of previously described methods (McDougall, J.K., Myerson, D., and Beckmann, A.M., "Detection of Viral DNA and RNA by In Situ Hybridization," J. Histochem. Cytochem. (1986) and Brigati, D.J., et al., "Detection of Viral Genomes in Cultured Cells and Parafin-embedded Tissue Sections Using Biotin-labeled Hybridization Probes," J. Virol., 126:32-50 (1983)). A positive signal (brown precipitate) was observed in the nuclei of the CaSki cells due to the presence of a HRP-avidin complex which binds to the biotinylated hybridized DNA probe. This enzyme converts aminoethylcarbazole to a colored product. Control experiments using biotinylated HPV-6, HPV-11,

and HPV-18 DNA as probe produced no color development, thus indicating that in situ hybridization was highly specific. Likewise, a negative control in situ hybridization of biotinylated HPV-16 DNA with A-549 cells, which contains no HPV-16, produced no color development within the cells.

Results with long biotinylated probes indicated that the CaSki cell nuclei had a distinctive signal pattern in the nuclei. Generally, there were 7-8 large brown granules per nucleus. Presumably, that each granule represents an HPV integration point within a unique chromosome, since CaSki cells are reported to contain about 700 copies of HPV-16 integrated in tandem arrays at 6-7 sites in the chromosomes. Thus, each integration site (i.e., each precipitate) roughly contains about 100 copies of HPV DNA.

Assuming 100 copies of HPV per granule, a probe dilution experiment was developed to test the sensitivity of our in situ hybridizations. Several dilutions of biotinylated HPV-16 DNA with non-biotinylated HPV-16 DNA were used in several in situ hybridizations with CaSki cells, and the point at which signal disappeared was determined. A sixfold dilution of the biotinylated HPV-16 DNA defined the limits of sensitivity, and this translated into a detection limit of 15-20 tandem copies of HPV-16 per cell (100/6=17).

In situ hybridizations with biotinylated HPV DNA probes can be conducted directly on stained and unstained cervical smear samples, using, e.g., biotinylated human placental DNA as probe. Total human DNA was chosen as probe, since this will hybridize rapidly with the highly repetitive DNA sequences of human cells, thus allowing optimal in situ hybridization conditions to be quickly defined. A detectable non-isotopic signal can be picked up during in situ hybridization with cervical smears using 10 ng of biotinylated human placental DNA.

Using this DNA probe, a preferred minimal treatment to achieve an in situ hybridization signal from cervical smear samples was derived in the following experiments.

Four cervical smear samples were taken by a gynecologist and immersed in 95% ethanol by the standard procedure:

- Sample 1 was processed directly.
- Sample 2 was immersed in Carnoy's B solution and processed.
- Sample 3 was fixed in paraformaldehyde, washed and processed.
- Sample 4 was fixed in paraformaldehyde, treated with Triton X-100, fixed again in paraformaldehyde, washed and processed (this procedure previously was developed for cervical smear in situ hybridization).

After annealing with biotinylated human placental DNA, these slides showed relative signals of 0.8, 1.0, 0.2, and 0.2 for Samples 1, 2, 3, and 4, respectively. These results suggest that pretreatments may be eliminated and cervical smears processed directly following the standard immersion in ethanol by the gynecologist.

Experiments were also conducted on in situ hybridizations at room temperature using biotinylated HPV-16 long probes and the CaSki cell line. Essentially no difference between the non-isotopic signal obtained at room temperature versus that obtained when conducting the assay at conventional temperatures (e.g., 37°C) was found. In addition, hybridization specificity is not lost at room temperature, since HPV-6 and HPV-18 probes produced no signal in the CaSki cell nuclei. These results indicate that rapid, specific and sensitive in situ assays may be formatted completely at room temperature.

In other experiments, in situ hybridizations were conducted on unstained cervical smears from the sexually transmitted disease clinic at Harborview Hospital in Seattle, Washington. Patients from this clinic show about 65% incidence of HPV infection. By in situ hybridization, analysis of unstained cervical smears from this population yielded the following results: 1) ten samples analyzed with a cocktail of HPV Types 6, 11, 16, and 18 produced five positive tests (60%) for HPV; 2) five samples analyzed with a cocktail of HPV Types 6 and 11 produced three positive tests for HPV; and 3) five samples analyzed with a cocktail of HPV Types 16 and 18 yielded one positive (20%). In nearly all of the positive tests, there was a small percentage of positive cells mixed with a much larger population of negative cells.

From the foregoing, it will be appreciated that the present invention provides a hybridization probe-based HPV diagnostic test that can be used in conjunction with traditional cellular smear methodology, particularly for identifying the presence and/or type of HPV in an infected sample of a cervical smear. The assays of the present invention are rapid, reliable and economical, and can be readily formatted for clinical laboratories.

Although the invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

1. A rapid in situ hybridization assay for detecting the presence of or typing Human Papillomavirus (HPV) in a non-frozen cellular smear fixed on a support in the absence of aldehyde-based cross-linking reagents, said assay comprising the steps of:

combining nucleic acids from the fixed biological sample with at least one detectable probe of about 50 or more nucleotides or analogs thereof, wherein the probe is capable of specifically hybridizing with a substantially complementary region from one or more HPV types; and

detecting the presence or absence of probe hybridization complexes, wherein the mixing and detecting steps are completed in less than about four hours.

2. An assay according to Claim 1, wherein the biological sample is fixed by treatment in an alcohol bath.

3. An assay according to Claim 1, wherein the support is a glass or plastic slide.

4. An assay according to Claim 1, wherein all of the steps are performed at between about 20°C and 37°C.

5. An assay according to Claim 1, wherein the cellular smear is obtained from a cellular lavage, scraping or biopsy.

6. An assay according to Claim 1, wherein the probe comprises nick-translated fragments.

7. A method according to Claim 1, wherein the hybridizing is homoduplexing.

8. An assay according to Claim 1, wherein the combining step is conducted in the presence of a tetraalkylammonium or triethylamine salt.

9. A method according to Claim 1, wherein the combining step is conducted in the presence of a volume exclusion agent.

10. An assay according to Claim 1, wherein said combining step is performed in less than about one hour.

11. An assay according to Claim 1, wherein all of said steps are performed in less than about two hours.

12. An assay according to Claim 1, wherein the HPV type is related to 6, 11, 16, 18, 31, 33, or 35.

13. An assay according to Claim 1, wherein the composition comprises at least two detectable probes, each probe being capable of specific hybridization with a different HPV type.

14. A method for diagnosing Human Papillomavirus (HPV) infections in a biological sample containing cells suspected of harboring integrated HPV, said method comprising the steps of:

treating the biological sample with a non-aldehyde based immobilizing agent, whereby DNA from the cells in the biological sample is fixed through the cells on a support under non-freezing conditions and made accessible to hybridization with nucleic acid probes;

combining under stringent hybridizing conditions the fixed nucleic acids and probes of 50 or more

nucleotides, or analogs thereof, substantially complementary to an HPV type;

washing to remove non-complexed probe; and

determining the presence or absence of DNA/probe complexes;

wherein the combining and determining steps are performed in between about 30 minutes and two hours and at least one of the steps is performed at room temperature under stringent hybridizing conditions.

15. A rapid in situ hybridization test for the detection and typing of Human Papillomavirus (HPV) in a cervical smear sample fixed on a glass slide in an ethanol solution, said method comprising the steps of:

(i) inactivating competing endogenous enzyme activity;

(ii) denaturing nucleic acids in the sample;

(iii) hybridizing a labelled probe to the target nucleic acids, wherein the probe comprises a sequence of at least about 50 nucleotides complementary to one HPV type DNA or mRNA;

(iv) washing the sample to remove unbound probe;

(v) incubating the sample with detection agents; and

(vi) visually inspecting the sample.

16. A kit for Human Papillomavirus (HPV) detection by in situ hybridization of alcohol-fixed cervical cell smears, said kit comprising a first probe reagent component comprising a biotinylated probe of at least about 50 to 600 nucleotides complementary to a nucleic acid sequence of HPV types 6 or 11 and/or a second biotinylated probe of about 50 to 600 nucleotides complementary to a nucleic acid sequence of HPV types 16, 18, 31, 33, or 35; and a denaturation reagent

for converting double stranded DNA in the cells to single stranded DNA.

17. The kit of Claim 16, further comprising an enzyme labelled with avidin or streptavidin and an enzyme substrate.

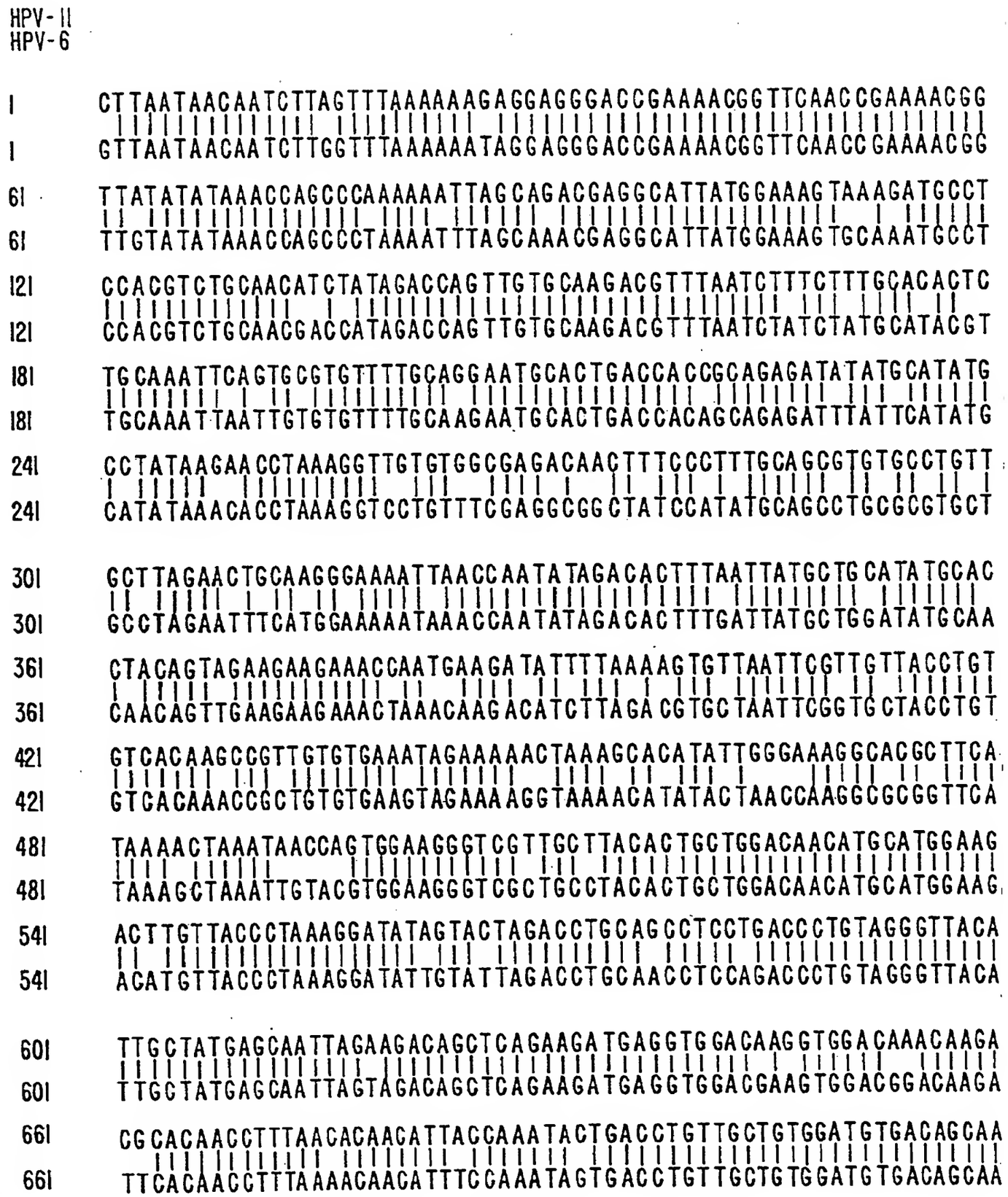

FIG.__1a.

SUBSTITUTE SHEET

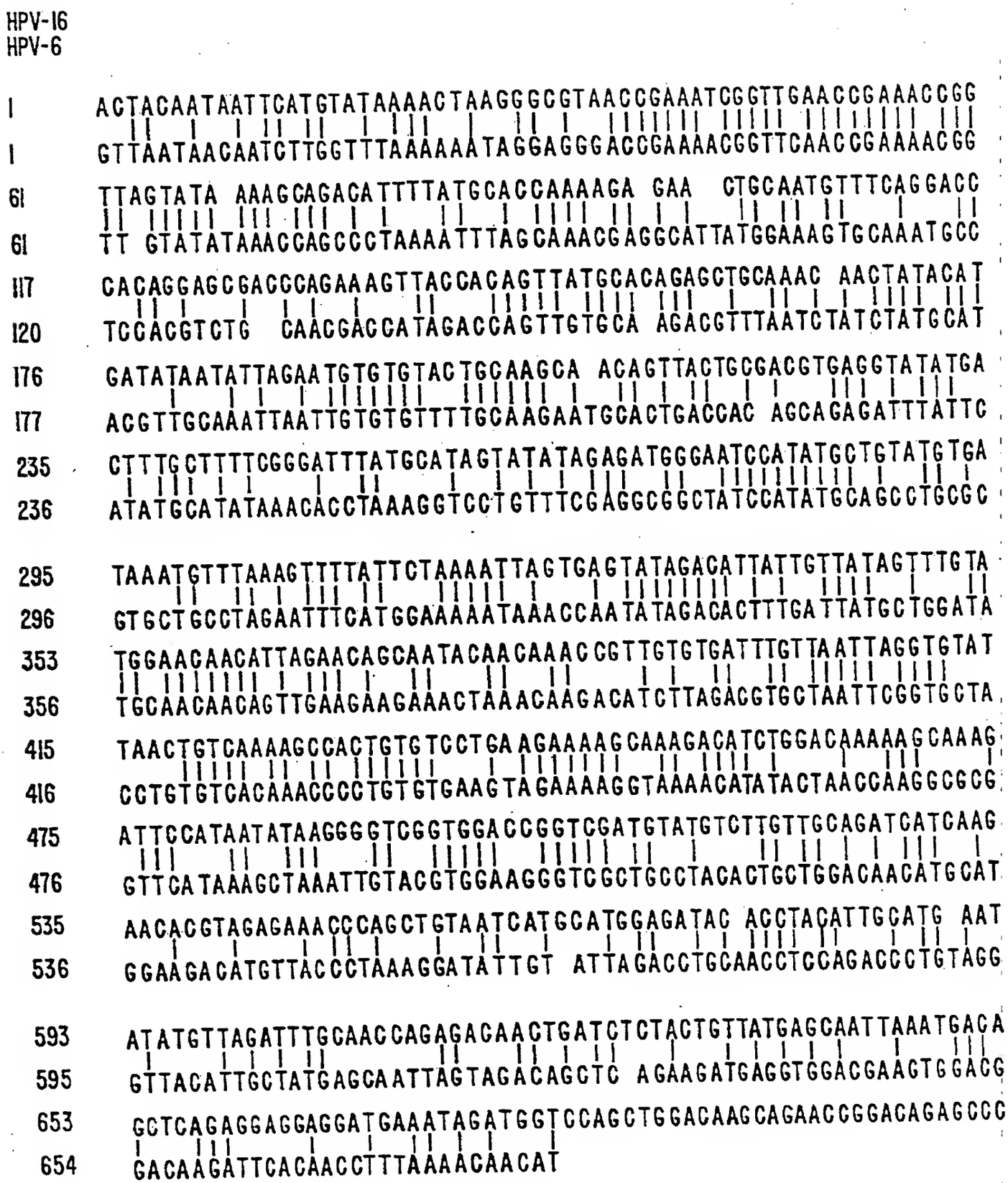

FIG. 1b.

SUBSTITUTE SHEET

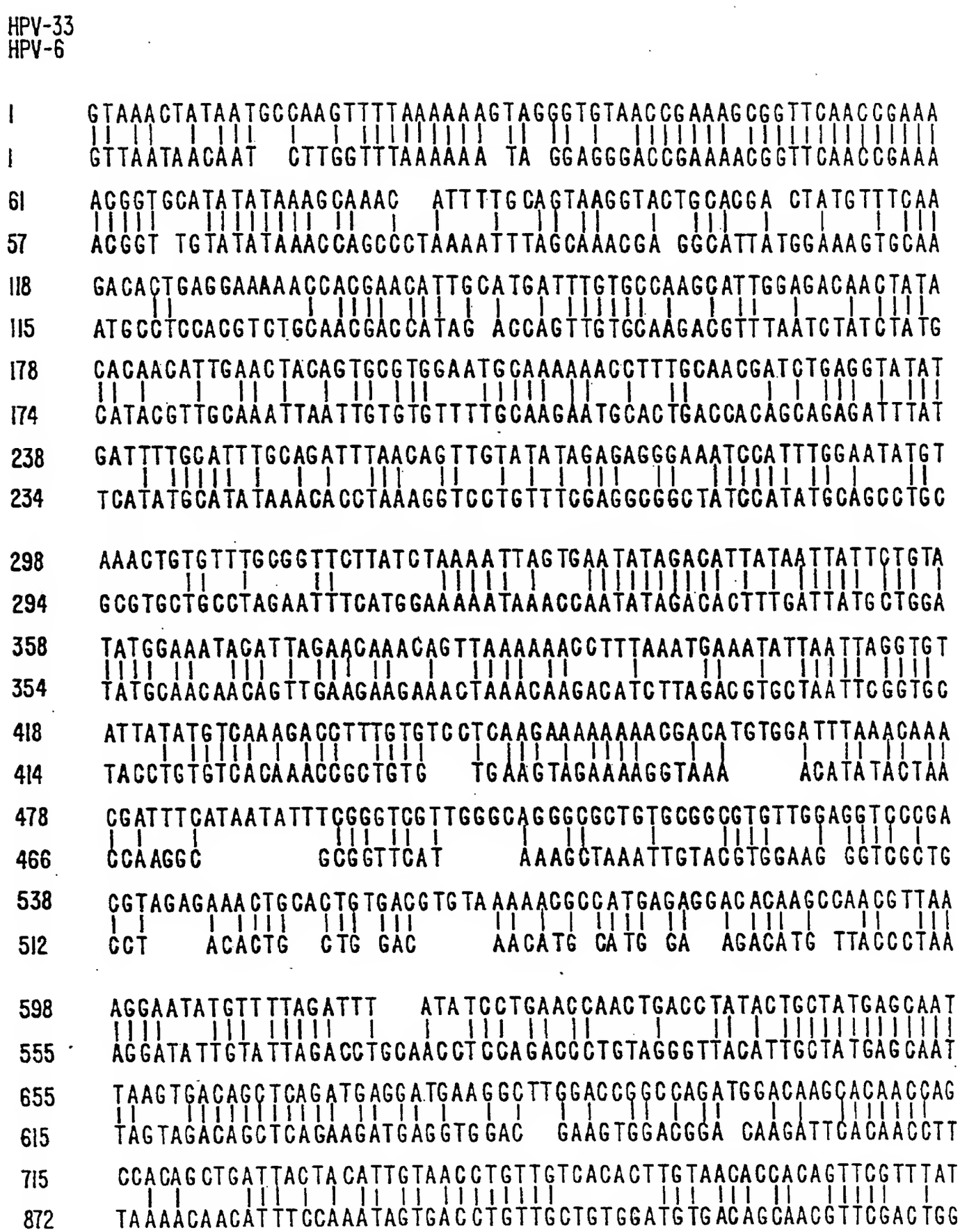

FIG._1c.

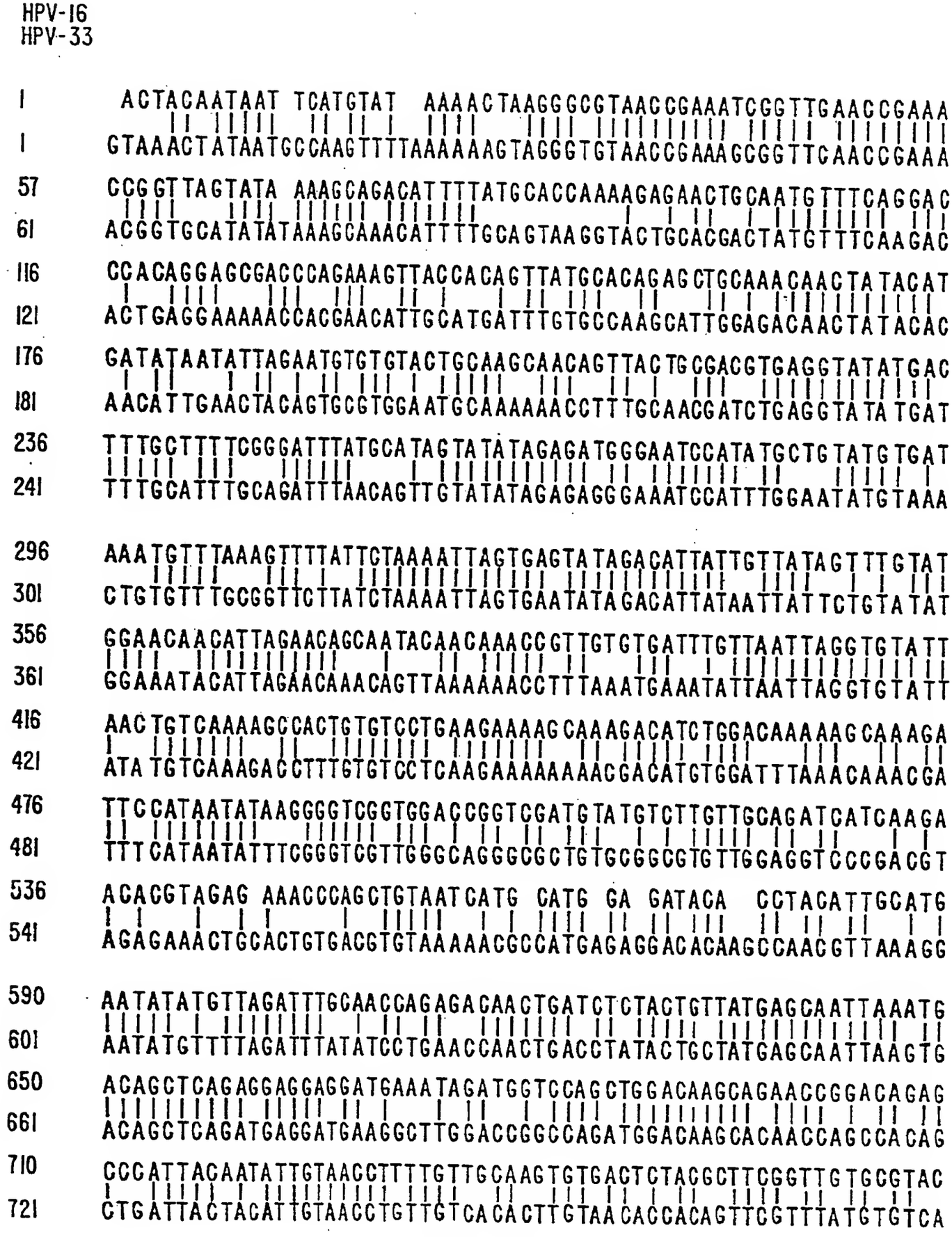

FIG._Id.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03367

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) C12Q 1/68, 70; G01N 33/53

U.S. CL.: 435/5,6,7, 810, 948; 436/501

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5, 6, 7, 810, 948 436/501

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	WO, A, 86/05816 (Georgetown University) 09 October 1986, see Abstract and Page 39.	16-17
Y	J. McDougall, et al, "Methods for Diagnosis Papillomavirus Infection in Papillomavirus, published 1986, by Wiley Chichester, (CIBA Foundation Symposium 120) (New York), see abstract, pages 91, 92, 96, 97.	1-17
Y	A. Hasse, et al., "Detection of Viral Nucleic Acids by In Situ Hybridization" in Methods in Virology, Volume VII published 1984, by Academic Press, Inc. (New York), see pages 199-200 and 223.	1-15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 December 1988	28 FEB 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	JACK SPIEGEL

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biotechniques, Volume 4, No. 3, issued 1986 March (Natick, MA), R. Singer, et al., "Optimization of In Situ Hybridization Using Isotopic and Non-Isotopic Detection Methods", pages 230-250, see page 238.	1-14
Y	Analytical Biochemistry, Volume 152, issued 1986 Feb., (New York), R. Amasino, "Acceleration of Nucleic Acid Hybridization Rate by Polyethylene Glycol", pages 304-307, see page 305.	1-14
Y	Proc. Natl. Acad. Sci. USA Volume 82, issued 1985 March (Washington, D.C.), W. Wood et al., "Base Composition-Independent Hybridization in Tetramethylammonium chloride: A Method for Oligonucleotide Screening of Highly Complex Gene Libraries", pages 1585-1588, see Abstract.	8
Y	Journal Medical Virology, Volume 16 issued 1985 March (New York) A. Beckmann, et al., "Detection and Localization of Human Papillomavirus DNA in Human Genital Condylomas by In Situ Hybridization With Biotinylated Probes", pages 265-273, see abstract and pages 266-267.	16-17
Y	Journal Oral Pathology, Volume 15, issued 1986 May, (Copenhagen), K. Milde, et al., "Detection of Papillomavirus DNA is Oral Papillomas and Carcinomas: Application of In Situ Hybridization with Biotinylated HPV 16 probes", pages 292-296, see abstract and page 295.	16-17